Phosphorylation site specificity of the CDC2-related kinase PITALRE

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PITALRE is a human protein kinase belonging to the cell division cycle 2 (CDC2) kinase family, and is the catalytic subunit of a multimeric complex that contains several cellular proteins. PITALRE complexes from several cell lines and tissues phosphorylate retinoblastoma protein and myelin basic protein (MBP). In the present work, we have found that MBP is phosphorylated by PITALRE complexes on both Ser and Thr residues. Two different antibodies raised to PITALRE purified virtually identical kinase activities, as analysed by MBP phosphopeptide mapping and phosphoamino acid analysis. We have

identified the proline-directed residue Ser-162 of MBP as a major phosphorylation site for PITALRE. In addition, our results suggest that one of the two MBP proline-directed threonine residues, Thr-97, is also selectively phosphorylated by PITALRE. These data, together with analysis of different peptide substrates derived from sites on MBP that are phosphorylated by PITALRE, indicate that PITALRE is a Ser/Thr proline-directed kinase. In addition, our results show that PITALRE has a substrate site specificity distinguishable from those of the CDC2 and cyclin-dependent kinase 2 (CDK2).

INTRODUCTION

Some members of the cell division cycle 2 (CDC2) family of protein kinases have been shown to play a crucial role in eukaryotic cell cycle control [1]. However, it has been demonstrated recently that some of these closely related kinases are involved in processes clearly distinguishable from proliferative functions [2]. Therefore, although sharing structural motifs, regulatory sites and similar mechanisms of activation, this family of protein kinases has a broad spectrum of functions.

One of the most fascinating aspects in the study of protein kinases is the identification of their physiological substrates. Since several protein kinases with clearly different functions act at similar consensus phosphorylation sites, the primary sequence of these sites does not determine absolute specificity [3,4]. However, useful clues about the physiological substrate for a specific kinase can be obtained by: (a) the identification of a consensus site; (b) a match between the activation time course of a kinase and the time course of phosphorylation of its putative substrate; and (c) the precise subcellular location of kinase and substrate at the time that phosphorylation takes place [1,3]. Several proteins have been proposed as cyclin-dependent kinase (CDK) targets, mainly as putative CDC2 substrates [3]. However, the overlapping activation times of some CDKs during the cell cycle and the similarity of their consensus phosphorylation sites makes it very difficult to identify unambiguously physiological enzyme–substrate pairs.

PITALRE is a human nuclear CDC2-related protein kinase which was identified in a PCR-based cDNA screening by means of its partial sequence identity with CDC2 [5]. PITALRE shares 40–47% amino acid identity with other members of the CDC2 family of protein kinases, and it is the catalytic subunit of a multimeric complex that phosphorylates the retinoblastoma protein (pRb) *in vitro* [6]. However, the kinase activity associated

with PITALRE does not appear to oscillate during the cell cycle [5].

Despite the sequence similarity among the CDC2-related protein kinases, clear differences have been found in their substrate specificity. Thus histone H1 and pRb are good substrates for CDC2 kinase and CDK2 *in vitro*. However, histone H1 is a poor substrate for CDK4, CDK6 and PITALRE, although these kinases phosphorylate pRb, and at least CDK4 and PITALRE phosphorylate myelin basic protein (MBP). CDC2 is a Ser/Thr proline-directed protein kinase [3], and both direct and indirect evidence supports the proposal that other members of this family are also Ser/Thr proline-directed protein kinases. For example, CDK4 phosphorylates pRb *in vitro* at sites identical to those found phosphorylated *in vivo* [7], and CDK5, the catalytic subunit of a brain proline-directed protein kinase, phosphorylates neurofilaments and the tau protein at Ser/Thr-Pro sites [2]. In contrast, CAK1 (CDK-activating kinase 1), another member of this family, phosphorylates Thr-161 of CDC2, which is not followed by a Pro residue; consistent with this, CAK1 does not phosphorylate histone H1 [8].

Since placing PITALRE within the CDC2-related protein kinase family does not conclusively implicate a clear substrate preference, the purpose of the present study was to map the phosphorylation sites of the PITALRE kinase activity on MBP and to characterize the sites of phosphorylation by PITALRE. Moreover, we sought to learn whether PITALRE belongs to the CDC2 subfamily of Ser/Thr proline-directed kinases or, in contrast, possesses a different phosphorylation site specificity.

EXPERIMENTAL

Cells, biological assays and reagents

HeLa and 293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cell lysates,

Abbreviations used: CDC2, cell division cycle 2; CDK, cyclin-dependent kinase; CAK, CDK-activating kinase; PITALRE-CT, PITALRE C-terminal peptide; MBP, myelin basic protein; pRb, retinoblastoma protein; TAG peptide, peptide derived from the simian virus large T-antigen (see Table 4 for names and sequences of other synthetic peptides).

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immunoprecipitations and kinase assays using bovine MBP as an exogenous substrate were performed essentially as described previously [5]. Peptides containing potential phosphorylation sites were also used as exogenous substrates in kinase assays. These kinase assays were performed as previously described, with the exception that after 20 min of incubation at 30 °C the reactions were terminated as follows. The reaction mixture was briefly vortexed and rapidly centrifuged (10000 g, 5 seconds), and 20 µl of the supernatant was spotted (avoiding the Protein A-Sepharose beads, which contain the immunopurified protein kinase complexes) on to P-81 discs (Whatman phosphocellulose). The discs were immediately immersed in 175 mM phosphoric acid and washed by shaking (7 × 5 min). Then the P-81 discs were rinsed in acetone and, once dried, radioactivity was counted in an LKB liquid scintillation counter. All peptide kinase assays were performed at least in duplicate for each peptide. Negative controls were obtained by performing identical immunoprecipitations in the presence of the antigenic competing peptide (10 µg). An estimation of the radioactivity non-specifically incorporated was obtained by performing the kinase assays in the absence of substrate. The sequences of the peptides utilized in these kinase assays are shown in Table 4, except for the peptide derived from the simian virus large T-antigen (TAg peptide: ADANHATPPKKRKVQDPKDF).

Affinity-purified anti-PITALRE antibodies were obtained as described [5]. Anti-(PITALRE C-terminal peptide) (anti-PITALRE-CT), anti-CDC2 and anti-CDK2 affinity-purified anti-peptide antibodies were from Santa Cruz Biotechnology.

Phosphoamino acid analysis and phosphopeptide mapping

Phosphoamino acid analysis of the phosphorylated substrates obtained in the kinase assays was performed essentially as described previously [9]. A portion of the digest containing approx. 1000 c.p.m. was spotted on a 20 cm × 20 cm plastic-backed cellulose TLC plate (Baker-flex; Thomas). Electrophoresis was performed in aqueous 0.5% pyridine/5% acetic acid at 500 V for 140 min. TLC plates were dried in a vacuum oven at 65 °C and sprayed with 2% ninhydrin in acetone.

For the phosphopeptide mapping, the phosphorylated substrates obtained in the kinase assays were resolved by SDS/PAGE (Protogel; National Diagnostics) and transferred to a poly(vinylidene difluoride) membrane (Immobilon-P; Millipore). Plastic-wrapped membranes were autoradiographed and the autoradiogram was used as a template to cut membrane pieces containing the phosphoproteins. The small pieces of membrane were blocked in 0.5% polyvinylpyrrolidone (average molecular mass 40 kDa)/100 mM acetic acid for 30 min at 37 °C [10]. The digestion was performed in 100 mM ammonium carbonate, pH 8.9, containing 0.1 µg/µl trypsin. The tryptic peptides were lyophilized, resuspended in water, lyophilized again and dissolved in aqueous 1% pyridine/10% acetic acid. Approx. 6000 c.p.m. was spotted on a TLC plate as before. Electrophoresis was performed in aqueous 1% pyridine/10% acetic acid for 2 h 30 min at 340 V at 0 °C. Chromatography in the second dimension was performed with butan-1-ol/pyridine/acetic acid/water (15:10:3:12, by vol.) [11]. TLC plates were exposed to X-ray film or to phosphorimaging plates. Synthetic peptides were phosphorylated *in vitro* by PITALRE and were purified by two rounds of HPLC using a Vydac C18 reverse-phase column (4.6 mm × 250 mm) under the conditions described below. The purified phosphopeptides were lyophilized and resuspended in 100 mM ammonium carbonate, pH 8.9, containing the indicated concentrations of trypsin.

Phosphorylation of MBP and tryptic digestion

Anti-PITALRE-CT antibody (4 µg) was used to immunoprecipitate PITALRE protein complexes from a protein lysate of 6×10^7 HeLa cells. PITALRE protein complexes bound to Protein A-Sepharose were incubated for 50 min in 100 µl of 20 mM Na-Hepes, pH 7.4, 10 mM magnesium acetate, 1 mM dithiothreitol, 20 µM ATP (1.1×10^5 c.p.m./pmol) containing 4 µg/µl MBP at 30 °C. Phosphorylated MBP was separated from the immunocomplexes by centrifugation/filtration through a Spin-X column (Costar). MBP was concentrated by using a 10 kDa cut-off Spin-X-UF column (Costar) and precipitated with 10 vol. of acetone (−20 °C) overnight on ice. The precipitated protein was air-dried and resuspended in 20 µl of 8 M urea/0.5 M sodium bicarbonate, pH 8.2, and diluted 1:4 with water as described [12]. Digestion was performed with 10 µg of trypsin for 4 h at 37 °C, and then an additional 10 µg of trypsin was added and digestion was allowed to continue overnight.

HPLC separation of tryptic peptides and sequencing

Tryptic peptides were loaded on a Vydac C18 (4.6 mm × 250 mm) reverse-phase column using a Waters 715 Ultra WISP sample injection system at a flow rate of 1 ml/min driven by a Waters 600E controller. The column was equilibrated with solvent A (0.1% trifluoroacetic acid in water), and peptides were eluted with a 100 min linear gradient of 0–90% solvent B (0.1% trifluoroacetic acid in acetonitrile). Peptides were detected by UV absorption at 215 nm with a Waters 490 detector. Fractions from radioactive peak I were pooled and concentrated (8.4×10^6 c.p.m.). An aliquot of this pool containing 2.9×10^6 c.p.m. was loaded on a Vydac C18 (2.1 mm × 150 mm) reverse-phase column with solvent A as described above. Peptides were eluted at a flow rate of 0.2 ml/min with an incremental gradient of 1%/min in solvent B for 15 min, followed by 15 min at 0.5%/min and then 15 min at 1%/min. Fractions 21–26 contained most of the radioactivity (2.6×10^6 c.p.m.).

Additional peptide purification was obtained by loading an aliquot of fraction 23 (5×10^5 c.p.m.) on a narrow-bore Vydac C18 reverse-phase column (2.1 mm × 150 mm) with solvent A as described above. The column was eluted at a flow rate of 0.2 ml/min with a linear gradient of 0–90% solvent C (0.25 M urea/50% propan-2-ol) over 100 min. Fractions 9–16 contained most of the radioactivity (4.7×10^5 c.p.m.).

The Porton sequencer running with the standard protocol, reagents and support disk was unable to extract the phosphorylated Edman degradation products from the reaction cartridge, making it impossible to determine the labelled amino acids. In order to locate phosphorylated amino acids, we adapted the procedure reported by Wettenhall et al. [13] to the Porton gas-phase sequencer. In the modified procedure, the peptide was bound covalently to a Sequelon AA membrane using the procedure given by the manufacturer (Millipore). Ethyl acetate (S1) was replaced by 90% methanol to ensure that the majority of the phosphorylated products were extracted. Several modifications were also made to the standard sequencer protocol, including a 10% increment in the S1 delivery time and 20% increases in the drying time after S1 delivery and after delivery to the conversion flask. Under the modified conditions the sequencer allowed collection of radioactively labelled phenylhydantoin-amino acids. Radioactivity released at each cycle was quantified in an LKB liquid scintillation counter. Additional sequencing cycles after the last residue of a particular peptide were performed, and no radioactivity over background levels was detected. Consistently, little radioactivity remained in the Sequelon AA membrane after the final sequencing cycle. This indicates that

phosphorylation of sites contained in a hypothetical longer contaminating peptide is very unlikely.

RESULTS AND DISCUSSION

CDC2-related protein kinases are a family of kinases that share 40% or more identity at the amino acid level in an approx. 30 kDa region spanning their catalytic subunits [5,14–16]. Although these kinases are classified as Ser/Thr-specific, some tyrosine kinases also share sequence similarity with CDC2 kinases. To ascertain the phosphoamino acid specificity of the PITALRE kinase, we performed phosphoamino acid analysis of MBP upon phosphorylation by this kinase. MBP has been demonstrated to be a very good substrate for some protein kinases, and was used successfully in the identification of the Thr site phosphorylated by the meiosis-activated protein kinase p44^{mpk} [12]. MBP and pRb are phosphorylated *in vitro* by CDC2 kinase, CDK2, CDK4 and PITALRE. Since MBP is a relatively small protein compared with pRb, is commercially available and contains several potential Ser and Thr phosphorylation sites, we decided to use it to map the phosphorylation sites of the PITALRE kinase and to ascertain whether PITALRE is a Ser/Thr proline-directed kinase.

PITALRE protein complexes were isolated by immunoprecipitation with either affinity-purified anti-PITALRE antibodies [5] or with affinity-purified polyclonal anti-peptide antibodies directed to the C-terminus of PITALRE [6]. Phosphoamino acid analysis of MBP after kinase assays with PITALRE protein complexes revealed similar amounts of phosphoserine and phosphothreonine, independently of the antibody used to purify the complex (Figure 1A). Phosphopeptide mapping indicated that both immunopurified complexes had similar specificities, with four spots appearing to represent the major phosphorylation sites (Figure 1B).

MBP phosphorylated *in vitro* was separated from PITALRE protein complexes and subjected to digestion with trypsin. The tryptic digest was resolved by HPLC on a C18 reverse-phase column under the conditions described in the Experimental section. The radioactivity of individual peptides was determined by liquid scintillation counting of aliquots corresponding to each fraction. Figure 2(A) shows the profile of peptide and radioactivity distribution. The majority of the radioactivity was contained in peak I, which was analysed further. Fractions 25–27, corresponding to peak I, were pooled, concentrated and

re-chromatographed under slightly different conditions (see the Experimental section), and A_{215} peaks were collected manually (Figure 2B). Fractions containing the highest proportion of the radioactivity (fractions 22 and 23; see Figure 2B) were divided into aliquots and used to perform phosphoamino acid analysis, phosphopeptide mapping and peptide sequencing. In addition, we quantified the radioactivity eliminated at each cycle of Edman degradation to precisely identify the positions of the phosphorylated amino acids.

Table 1 shows the sequences of the two peptides contained in fraction 22. The radioactivity released at each cycle indicates that a phosphoamino acid is present at the third position of the tryptic phosphopeptide. Phosphoamino acid analysis of this sample indicated the presence of phosphoserine but not phosphothreonine (results not shown). This is consistent with our inability to identify the expected Ser residue at position 3 of peptide 1 (S¹⁶⁰GXP¹⁶⁶), suggesting that it was modified. Since there is a non-phosphorylatable Pro residue at the third position in peptide 2 (Table 1), we can conclude that Ser-162 is one of the major phosphorylation sites for the PITALRE-associated kinase on MBP.

Phosphoamino acid analysis of fraction 23 indicated the presence of both phosphoserine and phosphothreonine residues (results not shown). Sequencing of this fraction indicated the presence of at least five peptides, and radioactivity was released at cycles 1 and 6 (results not shown). Since it was not possible to conclude unambiguously the identity of the phosphopeptide, we performed an additional HPLC purification step on this fraction by using a urea/propan-2-ol gradient as described in the Experimental section. As shown in Figure 2(C), radioactivity was associated with peak 10. Table 2 shows the sequence of the peptide contained in peak 10 and the radioactivity released at each cycle, again indicating the presence of phosphoamino acids at positions 1 and 6. The radioactivity released at position 1 is in agreement with phosphorylation at Thr-97 of MBP (T⁹⁷PPPSQ¹⁰⁴), but phosphorylation at this site does not account for the radioactivity released at cycle 6, which would correspond to the non-phosphorylatable Gln residue in this peptide. Analysis of the primary sequence of MBP indicates the existence of one tryptic peptide and four partially digested peptide products containing Ser at position 6. One of these partially digested peptides (D¹⁵⁷SRS¹⁶⁶GSPMAR¹⁶⁶) contains Ser-162 in position 6. Ser-162 has already been identified as a major phosphorylation site (Table 1). Although extensive analysis was

Figure 1 Characterization of MBP phosphorylation by PITALRE kinase

PITALRE protein complexes were purified by using two different anti-PITALRE antibodies. (A) Phosphoamino acid analysis of MBP was performed as indicated in the Experimental section. Lane 1, anti-PITALRE-CT; lane 2, affinity-purified anti-PITALRE. The mobilities of the phosphoamino acids are indicated on the left. (B) Phosphopeptide maps of MBP phosphorylated by PITALRE protein complexes purified as in (A). Major phosphopeptides are numbered.

Figure 2 HPLC of the tryptic digest of MBP phosphorylated by the PITALRE-associated kinase

MBP was phosphorylated and digested with trypsin. (A) Tryptic peptides were resolved by HPLC on a C18 reverse-phase column (4.6 mm $\times$ 250 mm) equilibrated with 0.1% trifluoroacetic acid in water. The peptides were eluted with a linear gradient of 0–90% acetonitrile containing 0.1% trifluoroacetic acid at a flow rate of 1 ml/min. Fractions of 1 ml were collected, and radioactivity in 20 μ l aliquots of each fraction was measured by liquid scintillation counting. The absorbance at 215 nm is plotted, and radioactivity is indicated (●). Peaks of radioactivity (I and II) are indicated by brackets. (B) Fractions corresponding to radioactive peak I (25–27) were pooled and loaded on a C18 reverse-phase column (2.1 mm $\times$ 150 mm) equilibrated with 0.1% trifluoroacetic acid, and the peptides were eluted as described in the Experimental section. Fractions were collected manually as A_{215} absorbance peaks. Absorbance peaks 22 and 23 are numbered. The total radioactivity (c.p.m.) per fraction is indicated (●). (C) Peptides from fraction 23 (see B) were loaded on a C18 reverse-phase column (2.1 mm $\times$ 150 mm) equilibrated with 0.1% trifluoroacetic acid and eluted with a urea/propan-2-ol gradient at a flow rate of 0.2 ml/min (see the Experimental section). Fractions were collected manually as A_{215} absorbance peaks. Absorbance peak 10 is indicated. The total radioactivity (c.p.m.) per fraction is indicated (●).

not undertaken with peak II of the original tryptic digest, sequence analysis of fraction 32 from that peak (Figure 2A) revealed the presence of another partially digested peptide ($\text{N}^{91}\text{IVTPRTPPPSQGK}^{104}$), with release of radioactivity at cycle 7, which agrees again with phosphorylation of Thr-97. These results, taken together, suggest that MBP residue Thr-97 is also a phosphorylation site for the PITALRE kinase (see below). Minor phosphorylation at other sites cannot be ruled out.

Since both Ser-162 and Thr-97 are followed by a Pro residue in the MBP sequence, we investigated the possibility that the kinase activity of PITALRE is proline-directed. For this purpose we synthesized the peptide $\text{R}^{159}\text{SGSPMAR}^{166}$ (designated SGSP), which reproduces the Ser-162 phosphorylation site (Ser residue underlined). As expected, PITALRE specifically phosphorylated the SGSP peptide (Table 3). Since this peptide contains two Ser residues in its sequence, a peptide was synthesized in which the first Ser residue, which is not followed by a Pro, was changed to

Table 1 Sequence analysis of peptides from fraction 22 after HPLC separation of tryptic digests of MBP phosphorylated by PITALRE

The amino acid sequence of each peptide was determined as described in the Experimental section. Xaa represents an amino acid that could not be identified (see the text). The radioactivity released at each cycle was quantified using a liquid scintillation counter. The presence of a phosphoamino acid is indicated by *.

Cycle	Radioactivity (c.p.m.)	Amino acid	
		Peptide 1	Peptide 2
1	3107	Ser	Thr
2	2288	Gly	Pro
3	32784*	Xaa	Pro
4	10340	Pro	Pro
5	5090	Met	Ser
6	2817	Ala	Gln
7	2530	Arg	Gly
8	2118	—	Lys

Table 2 Sequence determination of peptide from fraction 10 after HPLC separation of tryptic digests of MBP phosphorylated by PITALRE

The amino acid sequence of the peptide was determined as described in the Experimental section. The radioactivity released at each cycle was quantified using a liquid scintillation counter. The presence of a phosphoamino acid is indicated by *.

Cycle	Radioactivity (c.p.m.)	Amino acid
1	449*	Thr
2	224	Pro
3	137	Pro
4	142	Pro
5	152	Ser
6	579*	Gln
7	385	Gly
8	164	Lys

Ala: RAGSPMAR (designated AGSP). Interestingly, the AGSP peptide proved to be a better substrate than the original SGSP peptide. Table 3 shows that the substitution of Ser by Ala resulted in a several-fold increase in phosphorylation, as determined by incorporation of ${}^{32}\text{P}$. This suggests that other residues near the Ser-Pro site may be positive determinants in the PITALRE consensus site. The importance of the Pro residue was

Table 3 Analysis of the SGSP phosphorylation site

In vitro phosphorylation of the peptides SGSP, AGSP and AGSA by PITALRE was assayed as described in the Experimental section (for peptide sequences, see Table 4). Each peptide was tested at three different concentrations. PITALRE protein complexes were immunopurified by using affinity-purified anti-PITALRE-CT antibodies. The values shown (total c.p.m.) are the means of two duplicates in a representative experiment (values differed by less than 15%). This analysis was performed three times with similar results.

[Peptide] (μM)	${}^{32}\text{P}$ incorporation (c.p.m.)		
	SGSP	AGSP	AGSA
150	455	18683	0
300	1172	30941	19
600	2337	50007	0

Figure 3 Analysis of the phosphorylation site of TP peptides

(A) *In vitro* phosphorylation assay of the TP1 and TP2-RR peptides (300 μM) by PITALRE (see the Experimental section). The left-hand bar of each pair indicates the c.p.m. incorporated into the peptides; the right-hand bar indicates their negative control (see Table 3 for details). (B) Purification of TP1 and TP2-RR phosphopeptides by two rounds of HPLC upon *in vitro* phosphorylation by PITALRE. (C) Tryptic digestion map of HPLC-purified TP1 and TP2-RR phosphopeptides on using different concentrations of trypsin (indicated in μg above the lanes). The TP1 and TP2-RR peptide sequences are displayed. The arrows indicate sites of cleavage by trypsin; asterisks indicate the phosphorylated residue. The expected number of Arg residues in each phosphopeptide is shown. The lesser produced intermediate is designated by 2' (see the text for further details).

clearly demonstrated by a kinase assay performed with another peptide, RAGSAMAR (designated AGSA), in which the proline adjacent to the putative phosphate acceptor residue has been substituted by Ala. Table 3 shows that phosphorylation of the AGSP and the AGSA peptides, which differ in a single amino acid residue, i.e. Pro to Ala). Therefore a Pro residue adjacent to the Ser residue appears to be required for phosphorylation of the latter.

Since Thr-97 of MBP also appears to be phosphorylated by PITALRE, we synthesized the peptide V⁹³TPRTPPPSQ¹⁰² (designated TP1), derived from the MBP sequence containing Thr-94 and Thr-97. When we used this peptide as a substrate for PITALRE, we could not detect any radioactivity bound to the P-81 phosphocellulose discs (Figure 3A). Since this peptide only contains one Arg residue, it might not bind to P-81 efficiently. To test this possibility, we synthesized another peptide, VTPRTPPRR (designated TP2-RR), containing two Arg residues at the C-terminal end following the proline residues. Phosphorylation of TP2-RR was readily detected (Figure 3A). To ascertain whether the TP1 peptide is also a substrate for PITALRE, HPLC analysis was performed on both peptides (TP1 and TP2-RR) upon incubation with the PITALRE kinase in the presence of $[\gamma$ -

$^{32}\text{P}]\text{ATP}$. Phosphorylation of both peptides could be detected by measuring the radioactivity present in the HPLC fractions (Figure 3B). To ascertain which residue was phosphorylated, the phosphopeptides were concentrated and subjected to tryptic digestion using different concentrations of trypsin and analysed by TLC electrophoresis as described in the Experimental section (Figure 3C). During digestion of TP1, the single radioactive species corresponding to the phosphorylated peptide was degraded to yield a single, less cationic, phosphorylated species, consistent with cleavage at Arg-4 producing the non-radioactive sequence VTPR and the ^{32}P -labelled pTPPPSQ (pT is phosphothreonine). For TP2-RR, the single labelled species before digestion was degraded to produce two less cationic ^{32}P -labelled intermediate species, and a single less cationic final product. This behaviour is consistent with initial cleavage at Arg-4, giving rise to non-radioactive VTPR and $[\text{pT}]\text{pTPPPRR}$, followed by cleavage of the labelled peptide at Arg-9 to yield $[\text{pT}]\text{pTPPPR}$. Cleavage at terminal Arg residues is known to be slow [17], and the pattern of digestion, with lesser production of intermediate 2' ($[\text{pT}]\text{pVTPRpTPPPR}$) and slow cleavage of $[\text{pT}]\text{pTPPPRR}$ to the final product, helps to confirm the conclusion that only Thr-4 and not Thr-2 of this peptide is phosphorylated by PITALRE. In addition, the absence of a common final digested phosphopeptide in both sets of tryptic digests also indicates that Thr-2 (corresponding to Thr-94 in the MBP sequence) of both peptides is not phosphorylated (Figure 3C). The different migration positions of the undigested and the partially and totally digested phosphopeptides are consistent with the expected number of Arg residues present in each phosphorylated product. In agreement with this, the final phosphorylated product of the digested TP2-RR peptide, which should contain one Arg residue, migrates in a very similar fashion to the undigested TP1 phosphopeptide, which contains a single Arg residue. In addition, the final digested phosphorylated product of the TP1 peptide, which does not contain an Arg residue, migrates only slowly towards the anode.

Taken together, the results obtained with these two peptides are consistent with the identification of Thr-97 as a phosphorylation site for PITALRE on MBP. In addition, the lack of phosphorylation of Thr-2 in both peptides is in agreement with the absence of released radioactivity at the corresponding cycle during the sequencing of a tryptic peptide from MBP containing Thr-94 at position 4 (see above). Furthermore, Edman degradation of the *in vitro* phosphorylated peptide TP2-RR upon HPLC purification released radioactivity at cycle 5, but not at cycle 2 (results not shown), which further demonstrates that the Thr-5 of this peptide is the only phosphorylated residue. Interestingly, the lack of phosphorylation of Thr-2 (which is followed by a Pro residue) in these peptides suggests that residues near the Thr-Pro core site may act as negative determinants, or that specific residues N-terminal to the phosphorylation site may also be important for the phosphorylation of this site. The latter possibility is in agreement with the data obtained using the peptides derived from the Ser-162 site, where the importance of the N-terminal -2 residue was clearly demonstrated (Table 3).

We have shown previously that immunopurified PITALRE protein complexes phosphorylate pRb (p56^{Rb} or a pRb fusion protein with glutathione transferase) and MBP *in vitro*. However, under the same conditions histone H1 is inefficiently phosphorylated [5,6]. In this regard PITALRE appears to have a substrate specificity closer to those of CDK4 and CDK6 than to those of CDC2 and CDK2. The identification of the major sites of phosphorylation on MBP for PITALRE and the analysis of the substrate specificity of this kinase with synthetic peptides indicates that PITALRE has a clear preference for Ser/Thr proline-

Table 4 Phosphorylation of different peptides by PITALRE

The phosphorylation of different synthetic peptides by PITALRE was compared with phosphorylation of the SGSP peptide at a 300 μ M concentration (100%). The putative phosphate acceptor is underlined. The values shown (total c.p.m.) are the means of two duplicates of a representative experiment (values differed by less than 15%). This analysis was performed three times with similar results. p130-NT and PITALRE-CT peptides contain an additional Cys residue in the N-terminal region. p130-NT contains an additional Lys residue in the C-terminal region.

Peptide	Parent protein	Sequence	³² P incorporation	
			(c.p.m.)	(% of that in SGSP)
SGSP	MBP (residues 159–166)	RSGSPMAR	3957	100.0
AGSP	SGSP-based	RAGSPMAR	86726	2192.0
AGSA	SGSP-based	RAGSAMAR	100	2.5
TP2-RR	MBP (residues 93–102)	VTPRTPPRR	10881	275.0
p130-NT	p130 (residues 39–51)	CESPTQIQRFDEK	3828	96.7
PITALRE-CT	PITALRE (residues 353–372)	CSTNQSRNPATTNQTEFERVF	3765	95.0

Table 5 Differential phosphorylation of peptides containing Ser/Thr proline-directed sites by PITALRE, CDC2 and CDK2 kinases

The values shown (total c.p.m.) are the means of two duplicates of a representative experiment (values differed by less than 15%). This analysis was performed three times with similar results. The phosphorylation of each synthetic peptide was compared with the phosphorylation of the TAg peptide at a 100 μ M concentration (100%).

Kinase	Peptide	³² P incorporation	
		(c.p.m.)	(% of that in TAg peptide)
CDC2	TAg	64277	100
	TP2-RR	12586	19.6
	SGSP	71	0.1
CDK2	TAg	12064	100
	TP2-RR	1359	11.3
	SGSP	0	0
PITALRE	TAg	0	—*
	TP2-RR	4708	—*
	SGSP	1005	—*

* PITALRE does not phosphorylate the TAg peptide.

directed sites. In agreement with this specificity, a peptide derived from a region of the N-terminus of the p130 retinoblastoma-related protein (p130-NT), which contains Ser and Thr proline-directed sequences, was phosphorylated (Table 4).

Intriguingly, PITALRE itself becomes phosphorylated in *in vitro* kinase assays, and this phosphorylation is dependent on the activity of the PITALRE catalytic subunit [6]. However, no Ser/Thr-Pro sequences are present in the PITALRE amino acid sequence. It may be that this phosphorylation does not have physiological relevance. In this context, we have not been able to detect phosphorylation of PITALRE *in vivo* when 293 or HeLa cells are metabolically labelled with [³²P]P_i (results not shown). However, when a peptide resembling the C-terminus of PITALRE was used as a substrate for *in vitro* phosphorylation by PITALRE complexes, it became phosphorylated although none of the two Ser and four Thr residues present in the peptide are followed by a Pro residue (Table 4). Therefore it is possible that additional residues other than Pro may also allow for substrate recognition by PITALRE. In this regard the CAK complex, first identified because of its ability to phosphorylate Thr-161/160 of CDC2 and CDK2 (a non-Pro-directed site), is a component of the general transcription factor TFIIF. Appar-

ently, CAK has the ability to phosphorylate the C-terminal domain of RNA polymerase II [18,19], which is known to be phosphorylated at Ser-Pro sites. It is likely that, similarly to CAK, other CDC2-related kinases possess a dual site specificity. Further studies will be required to resolve this issue.

To analyse whether the substrate specificities of PITALRE and the CDC2 and CDK2 kinases can be distinguished further, we measured the ability of these kinases to phosphorylate the SGSP and TP2-RR peptides, and also the TAg peptide, which is known to be an excellent substrate for CDC2 [20,21]. This peptide contains a perfect CDC2 consensus site (Ser/Thr-Pro-Xaa-Lys/Arg), in which a basic residue (Lys) is placed at position +3 from the phosphorylatable Thr site. As shown in Table 5, both CDC2 and CDK2 phosphorylated the TAg peptide to several-fold greater extent than the TP2-RR peptide (5–9-fold) and more than 900-fold greater than for the SGSP peptide. Indeed, CDK2 does not phosphorylate the SGSP peptide. Interestingly, although the TAg peptide contains a Thr proline-directed site, followed by an additional Pro residue as in the TP2-RR peptide, PITALRE does not phosphorylate it, indicating that residues near the core site may act as negative determinants. In this regard the presence of a basic residue (Lys) at the +3 position may represent this negative determinant. In agreement with this result, the histone H1 protein, in which Ser/Thr proline-directed sites are followed by basic residues, is not a good substrate for PITALRE (results not shown). Furthermore, these data are also in agreement with phosphorylation of pRB by PITALRE, since various Ser/Thr-Pro sites not followed by basic residues are present in this protein. Moreover, Thr-94 of MBP and Thr-2 of TP2-RR, which are followed by Arg at position +2, were apparently not phosphorylated by PITALRE (see above). Altogether these results indicate that, although PITALRE has a clear preference for Ser/Thr proline-directed sites, the residues near the core site may act as positive or negative determinants. In addition, PITALRE has a substrate specificity clearly distinguishable from those of CDC2 and CDK2.

Finally, we have tested the suitability of peptides derived from identified phosphorylation sites in MBP in kinase assays using whole crude protein lysates. The results of these experiments indicate that other kinases whose substrate specificities overlap with that of PITALRE exist in the cell (results not shown). This was expected, since mitogen-activated protein kinases (MAP kinases) phosphorylate Thr-94 of MBP, and other Ser/Thr proline-directed kinases also phosphorylate MBP. In this respect, we are not aware of any report of synthetic peptides and/or other

protein substrates that are suitable for the specific measurement of the kinase activity of any particular member of the CDC2 family of protein kinases in crude protein extracts. Nevertheless, the use of the synthetic peptides SGSP, AGSP and TP2-RR as substrates should provide a simple and rapid means of measuring PITALRE kinase activity (see the Experimental section). The use of these peptides avoids the need for the time-consuming bacterial expression and purification of pRb, which was the preferred substrate for the measurement of PITALRE kinase activity prior to the present study. In addition, the use of P-81 phosphocellulose discs overcomes the requirement for time-consuming SDS/PAGE analysis.

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